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Pest and disease resistance enhanced by heterologous suppression of a *Nicotiana plumbaginifolia* cytochrome P450 gene *CYP72A2*

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Abstract

The functional role of the *Nicotiana plumbaginifolia* cytochrome P450 gene *CYP72A2* was investigated in transgenic plants. *N. tabacum* plants transformed with a sense or antisense *CYP72A2* construct exhibited diminished heights, branched stems, smaller leaves and deformed flowers. Western blot analysis revealed reduced levels of a 58 kDa protein corresponding to CYP72A2, suggesting that the *CYP72A2* homolog was suppressed in the sense and antisense plants. Transgenic plants had increased resistance to *Manduca sexta* larvae that consumed about 35% to 90% less of transgenic versus control leaves. A virulent strain of *Pseudomonas syringae* pv. *tabaci* induced a disease-limiting response followed by a delayed and decreased development of disease symptoms in the transgenics. *CYP72A2* gene mediated resistance suggests that the plant-pest or -pathogen interactions may have been modified by changes in bioactive metabolite pools.

Introduction

Plant cytochrome P450s form a large family of heme-containing monooxygenases that are involved in the synthesis of a variety of secondary metabolites that include hormones, sterols, fatty acids, plant allelochemicals and xenobiotics. Specific metabolic functions of cytochrome P450 monooxygenases have been difficult to identify because the enzymes are highly labile and in low abundance making purification difficult. Sequencing of plant genomes has facilitated the classification of some P450s into unique families with predicted functions. Individual P450s have been assigned specific functions using reverse-genetics, knock-out populations and overexpression of P450 genes in transgenic plants (Ito & Meyerowitz 2000, Feldmann 2001).

We cloned a P450 cDNA designated as *CYP72A2* (U35226) from *Nicotiana plumbaginifolia* plants that were transformed with an isopentenyl transferase (*ipt*) gene that regulates the synthesis of cytokinins (Mujer & Smigocki 2001). Elevated cytokinin levels in the *ipt* transformed plants correlated with increased insect resistance and induced the accumulation of insecticidal compounds (Smigocki *et al.* 1997). The deduced amino acid sequence of *CYP72A2* has 45% identity to the *CYP72A1* gene (L10081) that was cloned from a *Catharanthus roseus* cell culture line selected for high indole alkaloid biosynthesis and shown to code for secologanin synthase, an enzyme that converts loganin to secologanin (Imler *et al.* 2000). Monoterpene alkaloids synthesized via the secologanin branch of the pathway include camptothecin and vinblastine that are powerful anticancer drugs and vincristine, a compound with insecticidal activity.

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We demonstrated that *CYP72A2* expression is induced by mechanical wounding, insect chewing and exogenously applied cytokinin (Mujer & Smigocki 2001). In addition, the wound response was systemic and occurred more rapidly in response to insect attack, suggesting a possible role for this gene in plant defense responses. We also cloned a highly homologous (75%) *Lycopersicon esculentum* homologue of *CYP72A2* (AF249329) and demonstrated that its expression is regulated by circadian rhythm and wounding as in *N. plumbaginifolia*, but not by cytokinin (Bartoszewski *et al.* 2002). It is presumed that the *N. plumbaginifolia* and *L. esculentum* P450 clones hydroxylate a different substrate than the *C. roseus* secologanin synthase enzyme CYP72A1, as they share only 45% identity at the protein level. Therefore, to characterize the functional role of *CYP72A2*, the gene was reconstructed for constitutive over- and under-expression and introduced into *N. tabacum* plants. We report on the effects of *CYP72A2* gene suppression on plant development and resistance to *Manduca sexta* and *Pseudomonas syringae* pv. *tabaci*.

Materials and methods

Plant transformation

Nicotiana tabacum cv. Xanthi leaf disks were cocultivated with *Agrobacterium tumefaciens* strain EHA105 carrying the full length *CYP72A2* cDNA cloned behind the CaMV 35S promoter in the sense (p35S-p450) or antisense (p35S-ASp450) orientation in pCAMBIA1380-35S binary plasmid that carries the hygromycin phosphotransferase II marker gene for selection of transformed plant cells (Bartoszewski *et al.* 2002). The 5′ end of the *CYP72A2* cDNA fragment (corresponding to bp 198–878) was cloned in the antisense orientation (p35S-tASp450). The pCAMBIA1380-35S plasmid was used as a control for transformation. Transformed shoots were selected on 40 mg hygromycin B (HgB) l^{-1} (Sigma, 81% pure). Transgenic plants were propagated in the greenhouse and fertilized twice a month with Peters 20N:20P:20K (United Industries Corporation). HgB resistant T2 progeny were selected from seeds of independently derived T1 plants segregating 3:1 for HgB resistance.

Polymerase chain reaction (PCR)

Genomic DNA was purified as described by Mujer & Smigocki (2001). PCR was carried out in a reaction mixture with 50–100 ng of genomic DNA as template, 10 mM Tris/HCl (pH 8.3), primers (1 μM each), 200 μM of each dNTP, 1.5 mM $MgCl_2$, 0.001% (w/v) gelatin and 20 units ml^{-1} Amplitaq DNA polymerase (Perkin Elmer/Cetus) at 94 °C for 45 s, 55 °C for 45 s followed by 72 °C for 3.5 min for 30 cycles in a 100 μl reaction mixture. The following primer sequences were used: forward (35S promoter region) 5′CCGGA ATTCCATGGAGTCAAAGATTCAAATAGAG GACC3′; reverse for the sense 5′GGCAAGC TTCTAGTAGTAGCACGACTTACA3′; and reverse for the antisense 5′GGCAAGCTTTATA CAATATGGTGGTGGCCAAAGATGATA3′ constructs.

Western blot analysis

Cell-free protein extracts were prepared from 100 mg leaves powdered in liquid N_2, homogenized in 1 ml of 0.1 M sodium phosphate (pH 7.6) containing 15 mM 2-mercaptoethanol, 1 mM EDTA, 1 mM DTT, 1 mM PMSF and 15% (v/v) glycerol, and total protein determined (Mujer & Smigocki 2001). Proteins (20 μg per lane) were separated on 9% SDS-PAGE, transferred to PVDF membrane (Bio-Rad) and CYP72A2 proteins detected with Tropix Western Star system (Applied BioSystems). Polyclonal antisera (1:1000 dilution) raised against CYP72A2 synthetic peptides were the primary antibodies (Mujer & Smigocki 2001). Experiments were repeated three times.

Insect bioassays

Newly hatched *Manduca sexta* (tobacco hornworm) larvae were maintained on an artificial diet (Carolina Biological Supply Company). Plant bioassays were conducted as previously described using 3 or 5 excised leaf disks (1.5 cm^2) that were placed on water-moistened filter paper with a single second instar hornworm (Smigocki *et al.* 1997). For extract analysis, leaf disks from untransformed plants were coated with aqueous suspensions of the leaf extracts at 1 and 10 mg ml^{-1}. Non-polar extracts were prepared by briefly dipping leaves from mid- to late-flowering plants in methylene dichloride, rotary evaporation at 30 °C, and drying under a stream of N_2 (Smigocki *et al.* 1997). Feeding and mortality were recorded at 24 and 48 h. Experiments were done in reps of 4 and each experiment was repeated 3 times.

Pseudomonas *infections*

Pseudomonas syringae pv. *tabaci* 11528 Race O and *P. synringae* pv. *syringae* 61 were grown overnight in King's B medium (Dhingra & Sinclair 1995) and washed twice in water. Leaves were infiltrated with the bacteria at 10^7 to 10^8 cfu ml^{-1} and incubated in the greenhouse or placed in a growth chamber with a 16 h day (270 μml m^{-2} s^{-1})/8 h night photoperiod when detached leaves were used.

Results

Transgenic P450 plants

Transgenic *N. tabacum* cv. Xanthi plants carrying the *CYP72A2* gene in the sense or antisense orientation were generated. Although the p35Sp450 sense gene construct generated numerous shoots, only one transformed plant matured and produced a small number of seeds for further propagation and analysis (T2 plants 1.1, 1.2, 1.3, 1.4, 1.5). The sense plants had branched stems and at flowering were about 15% shorter than the untransformed or transgenic control plants (Figure 1a). They had smaller and narrower leaves and 10% higher specific leaf weights (g cm^{-2}) than the controls. Normal flower development was also affected (Figures 1b and c). The style and filament were either shorter or longer than that of the control flowers, and the dark anthers produced a low number of viable pollen that yielded few if any seeds (Figure 1b and c). A developmental conversion of filaments to petals was also observed (Figures 1b and c). Transformation with the antisense constructs p35S-ASp450 and p35S-tASp450 produced 11 and 7, respectively, independently derived T0 transformants from which T2 homozygous plants were selected for further analysis. Antisense plants exhibited a relatively normal phenotype but some growth and developmental abnormalities similar

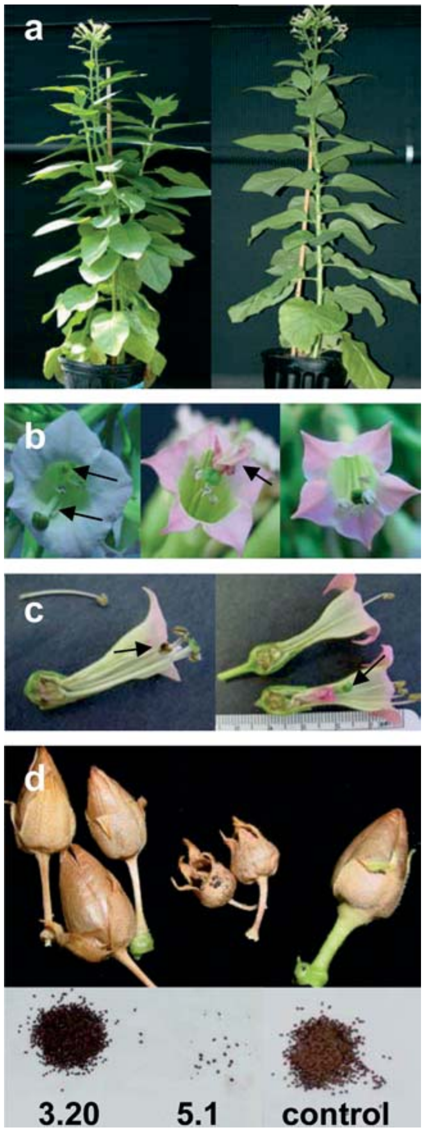

Fig. 1. Phenotypes of transgenic *CYP72A2 N. tabacum.* (a) Plant transformed with the sense construct (left) and control plant (right). (b) Flower of a sense plant exhibiting a long style (left) and filament to petal conversion (middle), and control flower (right). (c) Dark anthers (left) and short style and filament to petal conversion (right) of a sense plant. (d) Seed pods and seeds of full length 3.20 and truncated 5.1 antisense transgenic and control plants.

to those of the sense plants were detected (Figures 1b and d). Plants with the full length antisense construct had the least frequent occurrence of abnormalities. The seed pods were comparable to the controls except that some of the transformants carrying the truncated antisense *CYP72A2* gene resembled those of the sense plants in that the seed number per pod was low (Figure 1d, plant 5.1).

All transformed plants that were shown by PCR analysis to carry the introduced transgene (data not shown) were then analyzed for gene expression at the protein level using western blots (Figure 2). Plants transformed with the sense (1.1, 1.2, 1.3, 1.4, 1.5) or antisense (full length 3.1, 3.20, truncated 5.1, 5.5) gene construct had decreased protein levels of a 58 kDa polypeptide that corresponded to the predicted size of the CYP72A2 protein (Figure 2, lane 10; Mujer & Smigocki 2001).

Insect bioassays

Increased levels of insect resistance were observed with 5 of the 19 transformants that were bioassayed for resistance to tobacco hornworm larvae (Figure 3). The amount of leaf material consumed after 48 h ranged from 6% to 22% for mid- to late-flowering 1.1, 1.2, 1.3, 1.4 and 1.5 plants transformed with the sense construct of the *CYP72A2* gene (Figure 3a) as compared to almost 90% for the control. On average, about 60% and 30% consumption was observed of the full length (3.1, 3.20) and truncated antisense (5.1, 5.5) plants, respectively (Figure 3b and c). Approximately 30% of the larvae feeding on the sense transgenic plants died. At 1 mg ml^{-1}, surface extracts prepared from transgenic plants similarly reduced insect feeding and induced larval death (data not shown). Control extracts had no activity.

Pseudomonas *infections*

Infections with the avirulent *P. syringae* pv. *syringae* strain induced a disease-limiting hypersen-

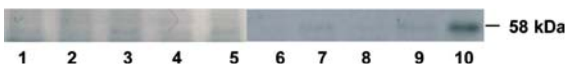

Fig. 2. Western blot analysis of total proteins from transgenic *CYP72A2 N. tabacum*. Plants transformed with the sense 1.1, 1.2, 1.3, 1.4, 1.5 (lanes 1–5) and antisense 3.1, 3.20 (lanes 6, 7), 5.1, 5.5 (lanes 8, 9) *CYP72A2* gene constructs and the *N. tabacum* control (lane 10). Proteins were reacted with CYP72A2 polyclonal antibodies. The expected 58kDa polypeptide is indicated.

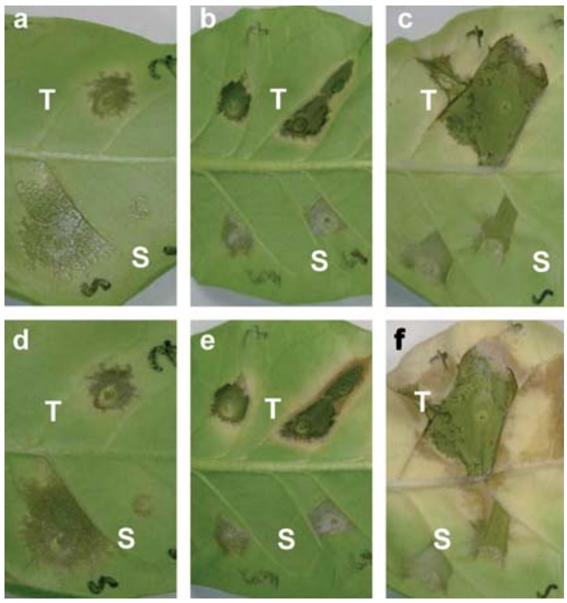

Fig. 4. Pseudomonas syringae infections of *N. tabacum CYP72A2* (a, d) 1.4 sense, (b, e) 5.5 truncated antisense and (c, f) control leaves at 6 (a, b, c) and 10 (d, e, f) days after inoculation. The virulent *P. syringae* pv. *tabaci* (T), showing chlorosis around the infection site, and the avirulent *P. syringae* pv. *syringae* (S), with no chlorosis around the HR lesion, were infused into the leaf on opposite sides of the midvein.

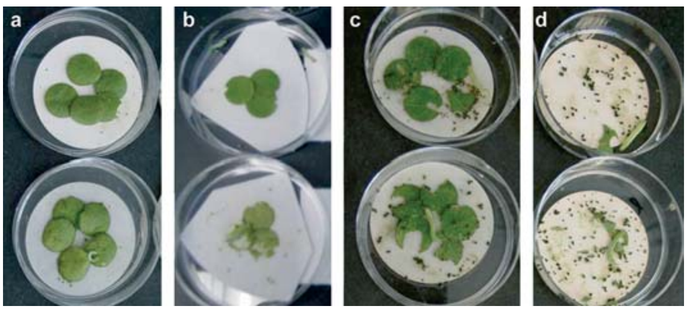

Fig. 3. Tobacco hornworm bioassay with leaf disks excised from *N. tabacum CYP72A2* (a) 1.4 sense, (b) 3.2 full length antisense, (c) 5.5 truncated antisense and (d) control plants. Five leaf disks were used except with 3.2 where 3 leaf disks were excised. Two replicate plates of each treatment are shown.

sitive response (HR) in both the transgenic and control plants (Figure 4, S). Infections with the virulent *P. syringae* pv. *tabaci* strain induced a disease-limiting response similar to HR in the transgenic plants as compared to disease symptoms on the controls (Figure 4, T). Tissue collapse and chlorosis were less severe in the transgenic plants at 6 and 10 d, and unlike the controls (Figures 4c and f), disease symptoms were limited to the perimeter immediately surrounding the infection site (Figure 4a, b, d and e).

Discussion

Heterologous expression and suppression of the *N. plumbaginifolia CYP72A2* gene was utilized to help elucidate its functional role. All progeny of transgenic plants exhibited similar phenotypic effects, however, they occurred less frequently in the antisense plants (Figure 1). Changes in shapes of flowers and seedless fruit were also induced when *CYP90C1* and *CYP78A9* were overexpressed in *Arabidopsis* (Kim *et al.* 1999, Ito & Meyerowitz 2000). In petunia and maize, abnormal pollen function and development were linked to reduced levels of flavanoids that are synthesized via cytochrome P450 pathways (van der Meer *et al.* 1992).

Expression of the sense *CYP72A2* gene construct had a deleterious effect on the survival of the transformed shoots that possibly could have been induced by a build up of toxic levels of secondary metabolites (Bak *et al.* 2000). Western analysis revealed that CYP72A2 protein levels were significantly reduced as compared to the control plants (Figure 2), suggesting that overexpression of the *CYP72A2* sense construct likely induced co-suppression of the endogenous gene. Co- and anti-sense suppression of the *N. tabacum CYP72A2* gene may have caused a build up of upstream precursor metabolites for which detoxification pathways may exist in *N. tabacum* or the build up may not have been as toxic to cell growth and survival. This is supported by the fact that numerous antisense plants were regenerated and that their phenotypes were similar to those of the progeny of the surviving sense plant.

Accumulation of secondary metabolites in plant cells can disrupt the interactions with pests and microbes by causing a direct effect on the pest or microbe and/or by altering the host cell physiology so as to render the compatible interaction incompatible or vice versa. The build up of precursor metabolites in the transgenic *CYP72A2* plants may have contributed to the observed increase in pest and disease resistance (Figures 3 and 4). Wang *et al.* (2001) demonstrated that suppression of a trichome gland associated *CYP71D16* by antisense and sense co-suppression strategies diminished aphid colonization responses due to a build up of a terpenoid precursor metabolite that accumulated at the blocked P450 enzyme in the transgenic plants. Several other cytochrome P450 genes have been identified that will be important for generating crop protectants and natural medicinal products (Feldmann 2001, Aharoni *et al.* 2003). *CYP82E1*gene cloned from HR lesions was shown to be strongly induced in tobacco by *P. syringae* pv. *glycinea* that is avirulent on tobacco, but it was activated only slightly and in a delayed fashion by a virulent *P. syringae* pv. *tabaci*, suggesting that CYP82E1 may play a role in disease resistance in tobacco (Takemoto *et al.* 1999).

This report documents both pest and disease resistance induced by heterologous suppression of a cytochrome P450 gene that may be mediated by a build up of bioactive precursor metabolites. Studies are in progress to identify the active compounds in leaf extracts of transgenic plants to assist with the elucidation of the corresponding biosynthetic pathways that are catalyzed by CYP72A2 to delineate their role in plant defense responses.

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